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TECHNICAL MANUSCRIPT 14

THE GROWTH OF ASIBI STRAIN YELLOW FEVER VIRUS IN TISSUE CULTURES

I. SENSITIVITY AND CAPACITY OF TISSUE CULTURES

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FOREWORD

The preliminary report that forms the basis for this series of papers was concerned with yellow fever virus growth in various tissue culture systems.[1]

Sensitivity comprises those factors concerned with the initiation of the infectious cycle at minimum multiplicities.

Capacity refers to the maximum detected virus concentration resulting from multiple infectious cycles.

ABSTRACT

A number of primary and established tissue cultures were examined for their susceptibility to yellow fever virus, with the most intensive study directed to HeLa cells. The following results were obtained: Of those cultures that were susceptible, three different patterns of dose response were obtained that were interpreted as different possible manifestations of interference. HeLa cells, which were among the least sensitive to low infection multiplication, had the greatest capacity (highest yields).

Subjecting cultures to wash cycles between 15 minutes and 2 hours post-inoculation increased the sensitivity (detectable virus) of such cultures 100-fold, increased the rate of virus multiplication, and had no effect on the peak titer (capacity). The HeLa cultures were found to be composed of different (morphological) clonal types. Three of these that were studied were all susceptible, comparable in capacity, but varied in their sensitivity.

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I. INTRODUCTION

The first study concerned with yellow fever virus in a tissue culture system was reported by Haagen and Theiler[2] in 1932. Of the various tissues that were surveyed for their susceptibility to the French (neurotropic) strain, chick embryo tissue proved to be most susceptible. In 1936 Lloyd and colleagues[3] indicated that minced mouse embryo tissue would support growth of the Asibi strain of yellow fever virus (a pantropic strain) and that chick embryo tissue would not. Eagle et al[4] in 1956 reported that a cell line (KB), derived from a human epidermoid carcinoma of the floor of the mouth, was susceptible to yellow fever virus. Hallauer[5] in 1959 has since confirmed the susceptibility of KB cells to Asibi strain virus.

The purpose of this paper is to report on the various tissue cultures that support the growth of Asibi strain virus and to point out factors that influence the rate of growth of this virus in one such cell culture system.

II. METHODS

A. VIRUS STRAIN

The Asibi strain of yellow fever virus as a 10% suckling mouse brain suspension was obtained through the courtesy of Dr. Max Theiler of the Rockefeller Institute of Medical Research. The second of two monkey plasma transfers and a final mouse brain passage were used for the preparation of master and stock seed pools, respectively, the latter having a titer of $10^{6.5}$ mouse intracranial (MIC) LD_{50}/gm of mouse brain. Infection of monkeys with the stock seed virus indicated that the virus had retained its established viscerotropic characteristics.

B. TISSUE CULTURES

Various monolayer cultures, prepared from primary tissues and established cell lines, were propagated in the yeast extract proteose peptone no. 3 medium of Mayyasi and Schurrmans.[6] Primary tissues used were monkey spleen, liver, heart, lung, and kidney. The established cell line cultures and their sources are listed in Table I.

C. WASH TREATMENTS

To insure the dilution of cellular and/or viral material after infection, a T-60 flask was subjected to four alternating cycles of removal and addition of medium. The removal was effected by asporation; the addition comprised a 20-ml volume of fresh medium. A total of 80 milliliters per flask were employed in the wash-treatment procedure.

TABLE I. ESTABLISHED CELL LINE CULTURES AND THEIR SOURCES

Tissue Culture	Source
Human amnion (HA)	Fogh and Lund (1957)[9]
Human heart (HH)	Girardi et al (1957)[10]
Human (embryo) lung (EHL)	Henle and Deinhardt (1957)[11]
Human cervix (HeLa)	Scherer et al (1953)[12]
Swine kidney (PK)	Harris (1959)[13]
Mouse lung (AML)	Isolated in our laboratories
Bronchosarcoma (MABEN)	Frisch et al (1955)[14]
Mouse fibroblast (L)	Sanford et al (1948)[16]

D. MOUSE TITRATION

The virus was titrated in Swiss albino mice and inoculated intracranially with 0.03-ml quantities of serial tenfold dilutions of virus. Ten mice were used for each dilution. The $MICLD_{50}$ endpoints were calculated by the method of Reed and Muench[7] and expressed as the concentration of virus contained in one milliliter. The deaths that occurred between the third and the fourteenth days, inclusive, were used in the final calculations.

III. RESULTS

A. STABILITY OF VIRUS

Since evidence for the growth of virus in tissue culture was to be based on increases in infectious activity contained in successive supernatant fluid samples, it was necessary to determine to what extent the tissue culture environment was deleterious to the infectious virus population. Thus, the inactivation of Asibi virus in tissue culture media at 37°C was tested.

Asibi strain virus was suspended in either medium 199[8] or yeast extract proteose peptone medium[6]; each of these media was supplemented with 20 per cent horse serum (HoS). Final concentrations of virus were adjusted to 10^4 $MICLD_{50}$/ml. All cell-free fluids containing virus were placed in a water bath at 37°C and sampled at two-hour intervals for eight hours.

The results showed that both basal media were deleterious to the virus; however, the incorporation of HoS appeared to retard markedly the very rapid titer decline. The rate of inactivation in either of the media supplemented with HoS approximated one log of infectious virus per four hours, which was comparable to the findings of Fox[16] with the 17D strain under similar conditions.

B. SCREENING OF TISSUE CULTURES

The first experiments were designed to test various tissue cultures for their susceptibility to the Asibi strain of yellow fever virus. Monolayer culture preparations were inoculated with virus and incubated at 37°C for six days. Paired tissue cultures were inoculated with 10^2 or 10^4 $MICLD_{50}$ doses, respectively. Samples were removed daily and titrated in mice.

Data that show the day post-inoculation when maximum virus titers were obtained are found in Table II. These results indicate that peak virus titers, under these conditions, occur at the 10^4 to 10^5 $MICLD_{50}$/ml level in all susceptible cell populations tested, with HeLa the only exception. HeLa showed no detectable virus growth after inoculation with the low dose, although titers as high as 10^7 $MICLD_{50}$/ml were obtained after inoculation with the high dose. Results of many trials with HeLa cells inoculated with Asibi strain virus showed that peak virus titers ranged between 10^6 and 10^7 $MICLD_{50}$/ml with the 10^4 inoculum; the 10^2 dose, however, always remained undetectable throughout the six-day period.

TABLE II. SCREENING OF TISSUE CELLS FOR THEIR SUSCEPTIBILITY TO YELLOW FEVER VIRUS

Tissue Cultures[a]	Peak Titers After Infecting With: 10^2 $MICLD_{50}$/ml		10^4 $MICLD_{50}$/ml	
Mouse kidney	3.0[b]	(4d)[c]	4.1	(2d)
Dog kidney	2.9	(2d)	4.5	(4d)
Duck (Peking) Fibr.			4.8	(2d)
L (Mouse Fibr.)	5.0	(5d)	3.4	(2d)
Mouse Lung	4.8	(5d)	3.6	(3d)
Human Amnion	3.3	(4d)	3.9	(6d)
Human Lung (Emb.)	5.8	(4d)	5.2	(5d)
Human Heart	2.9	(4d)	5.4	(3d)
Swine Kidney (PK)	3.5	(2d)	5.9	(3d)
HeLa	<2.0	(6d)	7.0	(6d)
HeLa (7th Passage Virus)			>8.0	(3d)

a. Monkey brain, heart, lung, liver, spleen and kidney (primary cultures) and Guinea pig lung, Human liver, and Maben (stable lines) were insusceptible.
b. Log_{10}.
c. Day peak titer was obtained.

Monkey tissue cultures were prepared as Maitland-type cultures without the aid of trypsin. Virus multiplication could not be detected in either the supernatant or the tissue fractions following inoculation. Virus multiplication was also undetected in trypsin-treated monolayer cell cultures of Maben and primary chick embryo cells. Microscopic examination of all cultures inoculated with the Asibi strain of yellow fever virus were negative for cytopathic effects.

When the data were grouped according to rates of virus growth initiated by low and high inocula, two general patterns were observed and a third indicated only the HeLa cell (Figure 1).

C. PATTERN I

Peak titers were found to be proportional to the virus concentration in the inoculum. Kidney tissue cultures of swine, mouse, and dog, and the heart and amnion human cell lines conformed to this pattern type.

D. PATTERN II

Peak titers produced were inversely related to the virus concentration contained in the inoculum. Established lung-cell cultures of the human, the mouse, and the L cell line responded to the virus in this manner. The configuration indicated by this type of virus growth suggested that auto-interference was responsible for the results, inasmuch as the greater inoculum served only to maintain a more or less static viral concentration, while the more dilute inoculum resulted in significant increases during the six-day period of incubation.

E. PATTERN III

A marked dose response, which was associated only with the HeLa cell culture, ranged from the maximum yield (Table I) to undetectable virus concentrations (Figure 1) following inoculation of 10^4 and 10^2 $MICLD_{50}$ doses, respectively. Thus, HeLa was shown to have the greatest capacity (ability to produce maximum virus concentrations) and yet the least sensitivity (ability to be infected at minimum virus concentrations) when compared with all other susceptible tissue cultures employed in these studies.

The slow rate of virus multiplication, however, was evident in all patterns when compared with that of a member of the group A arthropod-borne viruses, i.e., Venezuelan equine encephalomyelitis (VEE)[17] under similar conditions. The possibility was considered that the apparent slow rate of Asibi virus multiplication was due to its rapid rate of inactivation, which may conceivably have masked growth rates comparable with

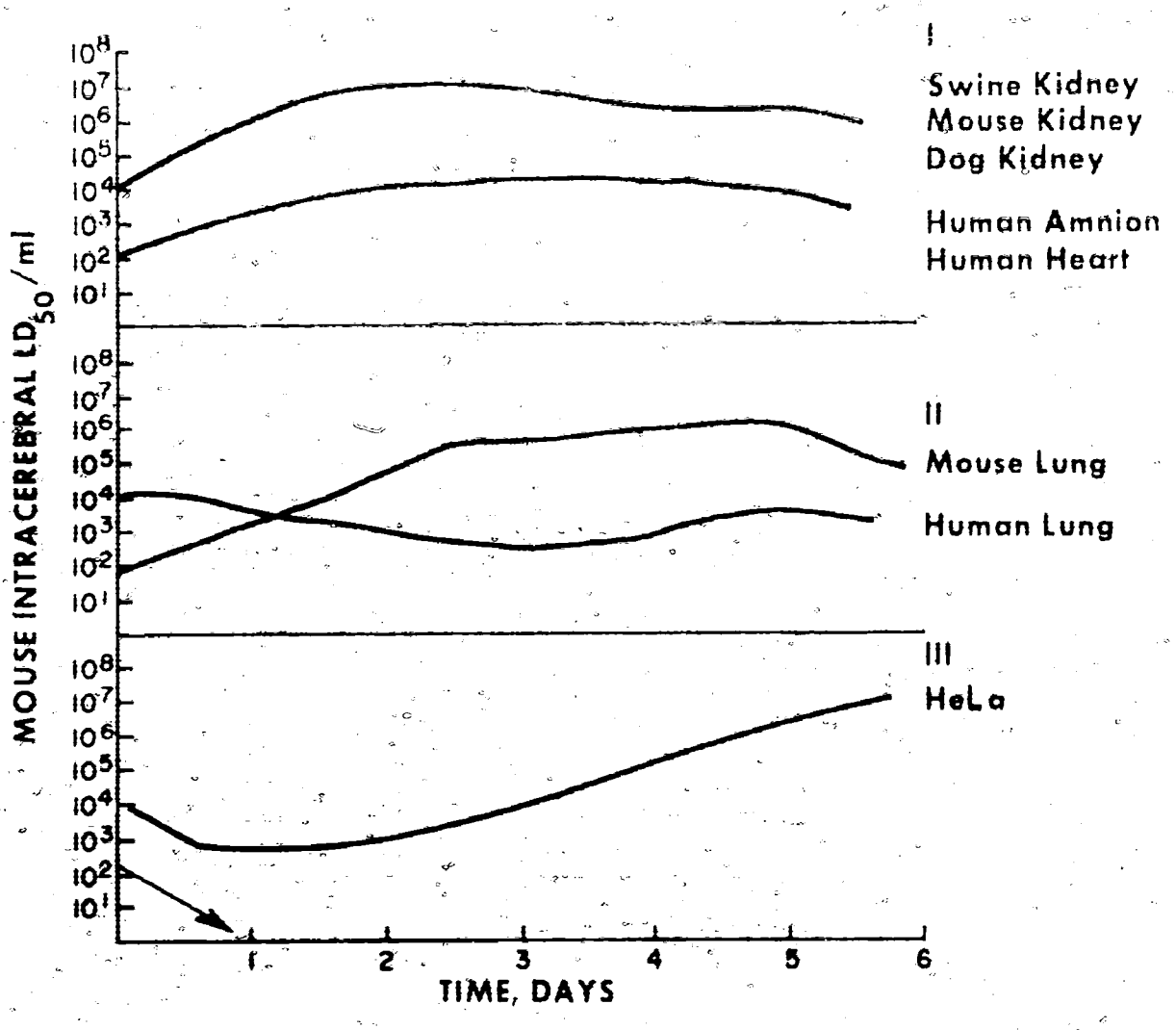

Figure 1. Patterns of Growth of Yellow Fever Virus, Asibi Strain, in Various Tissue Cultures.

those reported for the group A arthropod-borne viruses.[18-21] However, virus growth rates even in pattern I appear to indicate some degree of inhibition, inasmuch as peak virus concentrations achieved with the low dose were significantly less than those obtained with the high dose. The reciprocal relationship in the dose-response curve of pattern II has been accepted as a "classic" example of auto-interference.[22]

With respect to pattern III, however, it was of special interest that HeLa cells were apparently insensitive to low doses (10^2 $MICLD_{50}$), although their capacity to produce high virus titers when infected with the high dose (10^4 $MICLD_{50}$) was better than that of all other cell lines tested. Experiments detailed in a later section show that the apparent insensitivity of HeLa cells to the low virus dose was due to some type of interference.

If an interfering or inhibiting substance were present in the inoculum or cells could its concentration be reduced by repeated washing? A HeLa cell culture that was subjected to a single post-inoculation wash treatment with 10^4 $MICLD_{50}$ was compared with an unwashed similarly inoculated culture Results are expressed graphically (Figure 2) and indicate that with 10^4 $MICLD_{50}$ the rate of virus growth, from the time of inoculation until the attainment of peak titer, was related to the wash cycle during the early phase of virus growth. Peak titers were obtained from the culture treated with a single wash cycle in about half the time necessary for the unwashed culture. It was found in further experiments that no effect due to washing was observed beyond two hours post-inoculation.

In view of the results shown in Figures 1 and 2, an experiment was next designed to test the effect of washing on the sensitivity and capacity of the HeLa cells. Five HeLa cell flask cultures were subjected to a pre-inoculation wash cycle. Each culture was inoculated with a one-milliliter dilution of Asibi strain virus. The dilutions ranged in tenfold increments from 10^{-1} through 10^{-5} $MICLD_{50}$. All cultures were incubated at 37°C, and one hour later the excess inoculum was removed by washing. Supernatant fluids were removed from all cultures every 24 hours, and the cells washed with fresh medium. The supernatant fluids from these cultures and a HeLa cell culture inoculated with 10^4 $MICLD_{50}$, but subjected to a single post-inoculation wash treatment only, were titrated for virus content at daily intervals.

Samples obtained from HeLa cell cultures subjected to repeated washes pre- and post-inoculation throughout the six-day period were titrated (Figure 3). These curves showed that repeated washing, pre- and post-inoculation, increases the sensitivity of HeLa cells 100-fold, so that an inoculum containing $10^{2.2}$ $MICLD_{50}$ could be detected. No growth occurred with a $10^{1.2}$ inoculum. The capacity of HeLa cells in terms of virus yields was unchanged whether or not a wash cycle was performed (Figure 2). The size of the inoculum had no effect on capacity, nor on the rate of virus multiplication, but as with other viruses, multiplication started earlier and reached peak earlier with the highest inoculum.

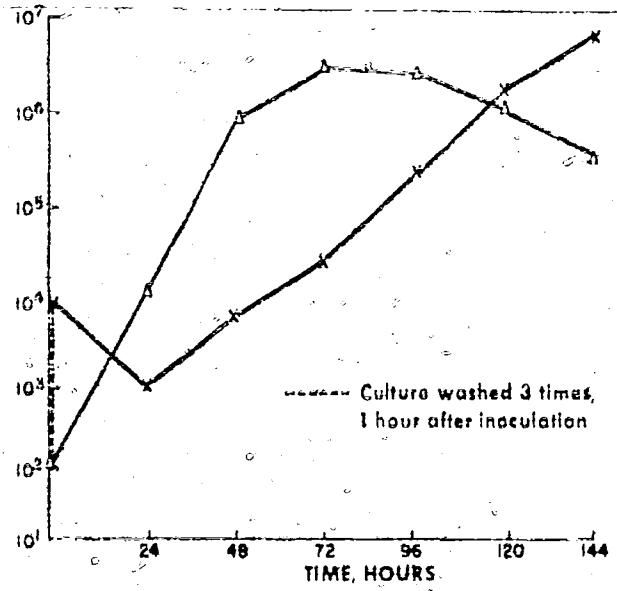

Figure 2. Growth of Yellow Fever Virus, Asibi Strain, in HeLa Cultures.

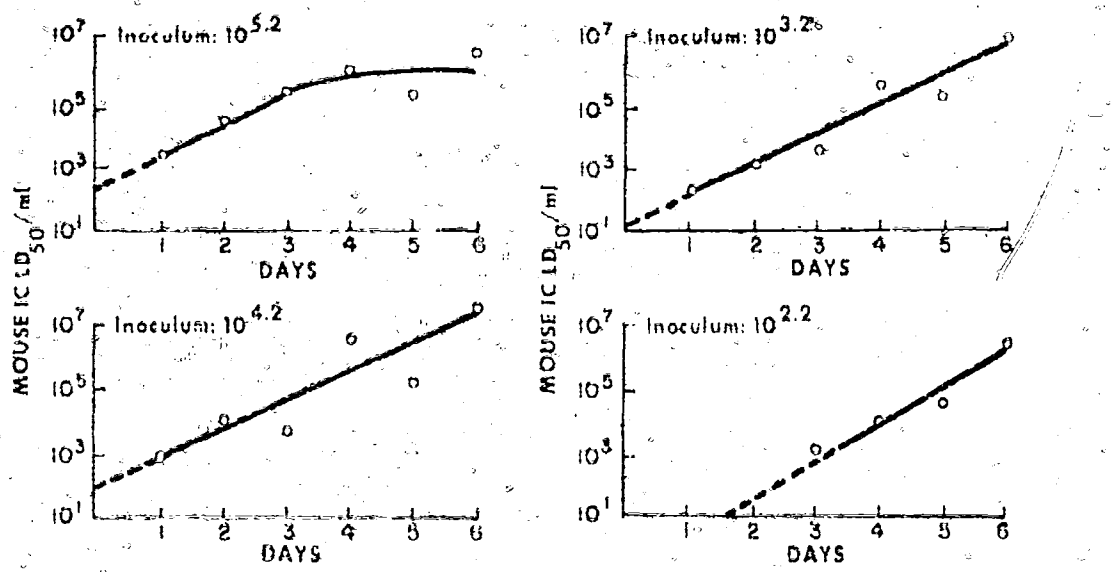

Figure 3. Exponential Growth Pattern of Yellow Fever Virus (Asibi Strain) in HeLa Cell Culture.

F. SUSCEPTIBILITY OF CLONES OF HeLa CELLS

Tests were made to determine whether HeLa cell cultures consist of a mixed population of varying susceptibility. Efforts were, therefore, directed toward obtaining different clonal types of cells and then to determine the rate of virus growth in those various clones. The parent HeLa population was cloned according to the method of Puck et al.[43] employing the yeast extract protoose peptone used in earlier experiments. Four morphologically different clonal types were observed in the first cell transfer. After clones were carried through two additional transfers to insure relative homogeneity (Figure 4) they were characterized as follows:

1. Clone A

Characteristically, this clone was of densely packed small cells that tended to grow laterally and vertically relative to the plating surface. Macroscopically, the clone had the appearance of an opaque white button. Clones of this type represented approximately 40 to 45 per cent of the population.

2. Clone B

Cells that comprised this clone were less densely packed than in clone A. The clone was comprised of large cells that tended to grow laterally over the plating surface. Macroscopically, this clone encompassed a larger area and was neither as opaque nor as convex as clone A. In addition, the frequency of isolation was usually 0.1 that of the clone A type and thus represented about four to five per cent of the population.

3. Clone C

Cells of this clonal type were sparsely scattered large cells, usually polar-connected, that spread eccentrically over the plating surface. Macroscopically, this clone appeared to be translucent. In normal parent populations of HeLa, clones of this type were isolated with difficulty, and represented about one to two per cent of the population.

4. Clone D

Microscopic examination of cells of this clone showed them to be relatively large and so thin that they were difficult to detect. They had no defined spatial arrangement. Unstained clones were not visible macroscopically, and transfer had to be effected with the aid of a microscope or by staining with neutral red. Clones of this type were most abundant and represented 45 to 55 per cent of the population.

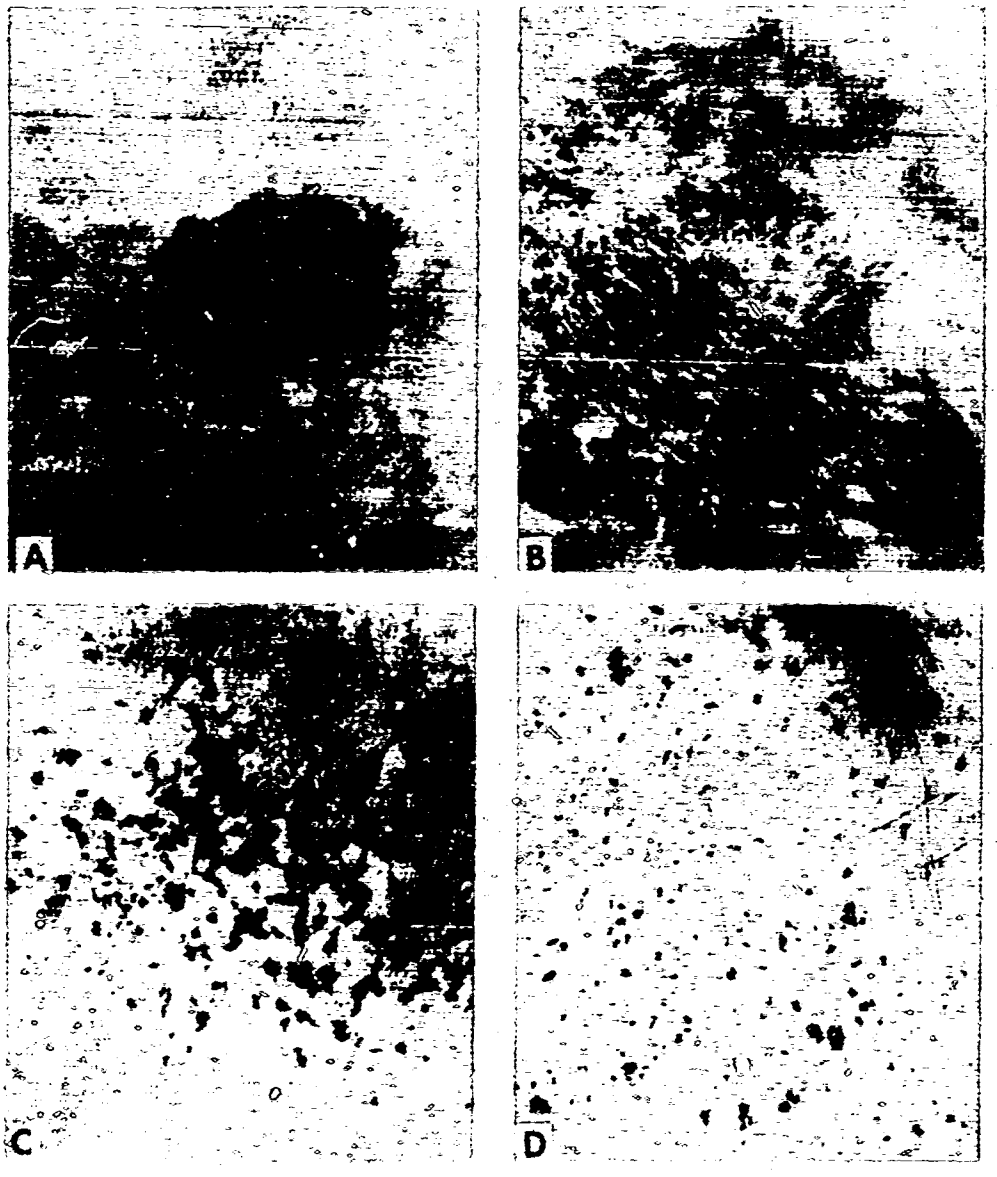

Figure 4. Clonal Isolates Derived from Parental HeLa Cell Populations (FD Neg C-6810).

Clones A, B, and D grown in T-60 flasks were tested for differences in susceptibility to Asibi strain virus. All flasks were inoculated with 10^4 $MICLD_{50}$ of Asibi strain virus and incubated for six days at 37°C. The cultures were not subjected to pre- or post-inoculation wash cycles. The results of titration with 0.5-ml daily samples indicated marked differences between the clones (Figure 5).

Clones A and D showed varying degrees of response to infection, which was reflected as an inability to demonstrate virus multiplication during the first three days of incubation. No virus was detected until the 120th hours in cells of clone A and 96 hours post-inoculation in clone D. On the other hand, considerable virus yields were obtained with clone B throughout the first three-day period, even though no effort was made to reduce the effects of interference. The peak virus growth in clone B occurred in half the time observed for the unwashed and uncloned cell population. Differences with respect to time were observed when one clonal element was compared with another. Thus, although sensitivity of the uncloned population is dependent on environmental manipulation (pre- and/or post-inoculation wash cycles), experiments with cloned populations indicate that sensitivity is a function of the host cell (e.g., B clone). The interaction by different clonal types in a mixed population are unknown.

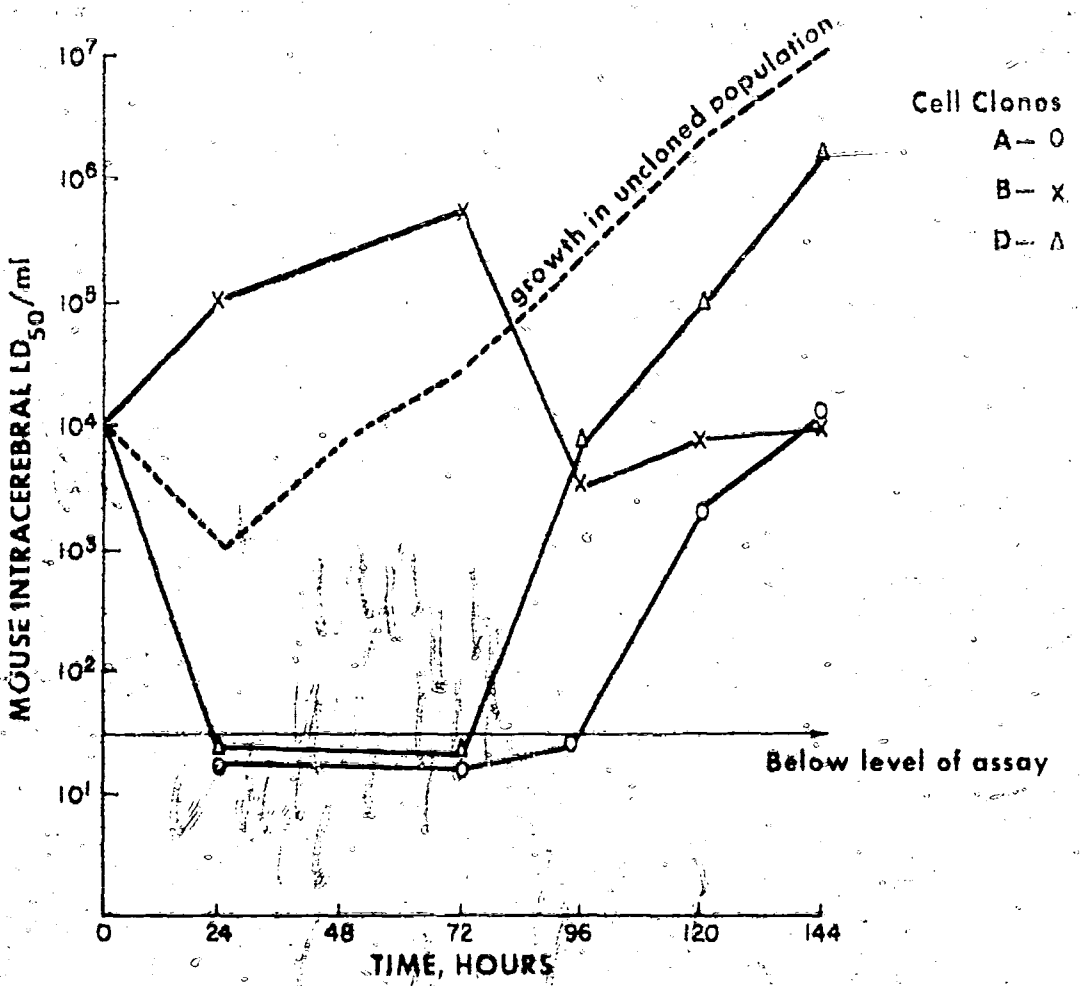

Figure 5. Growth of Yellow Fever Virus, Asibi Strain, in Three Clones of HeLa Cells.

IV. DISCUSSION

Various tissue cultures have been shown to have differing abilities to support the growth of Asibi strain yellow fever virus. The basic criterion of susceptibility was an increase in the concentration of virus in the supernatant fluid of such cultures subsequent to inoculation, regardless of magnitude. The results of dose-response and growth curves indicate that cultures may also be classified in terms of sensitivity and capacity as defined earlier.

A comparison of all susceptible tissue cultures tested showed that varying degrees of interference were manifest in these systems. In addition, HeLa cells were shown to have the greatest capacity for Asibi strain virus multiplication, but in contrast, proved to be the least sensitive (see pattern III) to the virus under test conditions.

The virus growth in cells under pattern II differed from pattern I in that the former represents classic auto-interference, while the latter dose-response curve appears associated with either of two possibilities, both of which were based on titers contained in serial samples of supernatant fluid: (a) Cell populations less susceptible to progeny (HeLa cell derived) than to parent (seed) virus; or (b) the existence of an inhibitor substance of cellular origin that retarded the infectious cycle. The low inoculating dose of pattern I never resulted in the comparable peak titer levels of the high dose.

Pattern III (HeLa) suggested an interference system different from the other two. The HeLa cells showed the greatest capacity to support the growth of Asibi strain virus, but they were relatively insensitive to low multiplicities. Thus, with HeLa the existence of an interfering or inhibitor substance upon virus infection appears highly probable. Sensitivity of HeLa to Asibi strain virus was shown to increase with dilutions of the inoculum and supernatant fluid by repeated washing up to two hours post-inoculation.

The results obtained in a survey of the sensitivity and capacity of untreated (unwashed) HeLa cell clones indicated that if an interfering or inhibitor substance were responsible, it was clone-specific. Each clone inoculated showed different sensitivities based on the time required for detection of virus multiplication. The capacity, however, to propagate Asibi strain virus of all clones tested was comparable. Since clone B cells showed a marked sensitivity to Asibi strain virus and the cells of either clone A or D did not, and since these clones represent at least three of the elements that comprise the total HeLa cell population, it is reasonable to believe that the varied dose-response sensitivity (10^2 *vs* 10^4) observed with the parent HeLa cell population was due for the most part to the cellular components of the HeLa population, and less to viral effects of the inoculum.

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